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Identification of a Feline Leukemia Virus Variant That Can Use THTR1, FLVCR1, and FLVCR2 for Infection▽

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The pathogenic subgroup C feline leukemia virus (FeLV-C) arises in infected cats as a result of mutations in the envelope (Env) of the subgroup A FeLV (FeLV-A). To better understand emergence of FeLV-C and potential FeLV intermediates that may arise, we characterized FeLV Env sequences from the primary FY981 FeLV isolate previously derived from an anemic cat. Here, we report the characterization of the novel FY981 FeLV Env that is highly related to FeLV-A Env but whose variable region A (VRA) receptor recognition sequence partially resembles the VRA sequence from the prototypical FeLV-C/Sarma Env. Pseudotype viruses bearing FY981 Env were capable of infecting feline, human, and guinea pig cells, suggestive of a subgroup C phenotype, but also infected porcine ST-IOWA cells that are normally resistant to FeLV-C and to FeLV-A. Analysis of the host receptor used by FY981 suggests that FY981 can use both the FeLV-C receptor FLVCR1 and the feline FeLV-A receptor THTR1 for infection. However, our results suggest that FY981 infection of ST-IOWA cells is not mediated by the porcine homologue of FLVCR1 and THTR1 but by an alternative receptor, which we have now identified as the FLVCR1-related protein FLVCR2. Together, our results suggest that FY981 FeLV uses FLVCR1, FLVCR2, and THTR1 as receptors. Our findings suggest the possibility that pathogenic FeLV-C arises in FeLV-infected cats through intermediates that are multitropic in their receptor use.

Feline leukemia viruses (FeLVs) are pathogenic retroviruses of domestic cats that induce proliferative, degenerative, and immunosuppressive disorders (17, 18, 27). Infected cats often contain a mixture of FeLVs that have been categorized into subgroups A, B, and C based on their interference and in vitro host range properties (39). These subgroups have been shown to be tightly associated with distinct feline diseases (17, 27). A fourth subgroup (T) that is highly related to FeLV-A has also been reported (26) and has been shown to be associated with immune deficiency (30). FeLV-A is the primary strain that is transmitted between cats and is the progenitor virus from which other subgroups arise. FeLV-B arises by recombination between endogenous retroviral sequences present in the cat genome and the gene encoding the FeLV-A envelope (Env) protein (32, 40, 42) that is responsible for host cell surface receptor recognition. FeLV-C and FeLV-T are formed by mutations in the FeLV-A Env gene (10, 27, 38). Env recombination and mutations are a major determinant of the host receptors used by the different FeLV subgroups (3, 23, 34, 46, 47).

The emergence of pathogenic FeLV-C in cats infected with FeLV-A provides a classic example of Env mutations that switch the host receptor used for infection, leading to a fatal pathogenic disease in the host. The emergence of FeLV-C is tightly associated with red blood cell aplasia, a fatal feline anemia characterized by a specific disruption in erythroid progenitor cell development (2, 12, 19, 28). Moreover, FeLV-C emergence coincides with a switch in the host receptor used for infection from the thiamine transporter THTR1 (FeLV-A receptor) (23) to the heme exporter FLVCR1 (FeLV-C receptor) (34, 35, 46). Previous studies have suggested that the feline anemia is caused by the FeLV-C Env protein binding to, and disrupting, the cellular function of FLVCR1 (1, 34, 46). Indeed, expression of FeLV-C Env in hematopoietic stem cells (36) or disruption of FLVCR1 (21, 35) specifically disrupts early erythropoiesis, which mimics the anemia observed in cats with FeLV-C. Interestingly, the emergence of FeLV-C from FeLV-A is analogous to the emergence of cytopathic X4 strains of human immunodeficiency virus type 1 (HIV-1) in individuals infected with the R5 strain of HIV-1. Analogous to the emergence of FeLV-C, X4 HIV-1 arises by Env mutations in the R5 HIV-1 strain, which leads to a switch in the coreceptor used for infection allowing an expansion in HIV-1 cell tropism and subsequently to accelerated AIDS (4, 8). However, whereas HIV-1 pathogenesis also involves emergence of variants or X4/R5 intermediates that can use multiple related coreceptors for infection (5, 8, 11), the mechanism of how FeLV-C emerges, in terms of the presence of FeLV-A/FeLV-C intermediates, has yet to be elucidated.

To better understand the emergence of pathogenic FeLV-C in infected cats and potential FeLV variants/intermediates that may arise, we isolated and characterized FeLV Env sequences from a primary FeLV isolate derived from a cat with pure red cell aplasia. In this study, we report the isolation and charac-

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terization of the novel FY981 Env, a hybrid FeLV-A/FeLV-C Env that, when pseudotyped, can use FLVCR1, THTR1, and the FLVCR1-related FLVCR2 for infection. Our findings suggest that the FY981 Env could represent a potential FeLV intermediate that arises during the emergence of pathogenic FeLV-C.

MATERIALS AND METHODS

Cells and viruses. Murine *Mus dunni* tail fibroblast (MDTF) (ATCC CRL-2017), TELCeB6 (9), feline kidney HO6T1 (29), feline fibroblast FEA (20), mink Mv1Lu (ATCC CCL-64), guinea pig 104C1 (ATCC CRL-1405), human HeLa (ATCC CCL-2), and porcine ST-IOWA (ATCC CRL-1746) cells were all maintained in Dulbecco's minimal essential medium with low glucose (Invitrogen Corp., Grand Island, New York, NY) and supplemented with 10% fetal bovine serum (FBS) (PAA, Etobicoke, Ontario, Canada). Chinese hamster ovary (CHO) cells (ATCC CCL-61) were maintained in alpha-minimal essential medium supplemented with 10% FBS. Human embryonic kidney cells, HEK293 (ATCC CRl-1573), and Phoenix ampho retroviral packaging cells (provided by Garry Nolan, Stanford University, Stanford, CA) were maintained in Dulbecco's minimal essential medium with high glucose and 10% FBS. TELCeB6 cells are retrovirus-packaging cells that do not contain retroviral envelope genes but produce noninfectious virus (9). Phoenix ampho cells produce replication-defective amphotropic murine leukemia virus (MLV).

The FY981 primary isolate was previously isolated from an anemic cat from Great Britain in 1980 (O. Jarrett, unpublished data). By using viral interference, the FY981 primary isolate was characterized as containing a mixture of subgroups A, B, and C and was shown to be anemogenic in neonatal cats (Jarrett, unpublished).

Expansion of primary FY981 FeLV isolate and amplification of FeLV envelope cDNA. Nonrestrictive feline FEA cells (1×10^5) were infected with 2 ml of primary FY981 FeLV isolate in the presence of polybrene (8 μg/ml) (Sigma-Aldrich, St. Louis, MO). Supernatant samples of the infected FEA cells were collected at regular intervals and assayed for FeLV p27 by enzyme-linked immunosorbent assay using a p27 detection kit (FeLV p27 ELISA; Idexx Laboratories Ltd., Buckinghamshire, United Kingdom). After detection of p27 in culture supernatant, total cellular genomic DNA was isolated from the transduced FEA cells (QiaAmp DNA blood minikit; Qiagen, MA) and used for amplification of the FeLV envelope gene.

The FeLV envelope gene was amplified from the isolated genomic DNA by PCR using the FeLV Env upstream primer 5-GCGTCGACCACCATGGAAA GTCCAACGCACCCAAAAC-3′ containing an SalI restriction site and the downstream primer 5′-CCGCGGCCGCAATCATGGTCGGTCCGGATCGTA TT-3′ containing an NotI restriction site. Amplified products were initially cloned into the pCR1 cloning vector (Invitrogen) and sequenced using an ABI Prism 3100 sequencing machine. Novel FeLV Env sequences were then subsequently cloned into a SalI-NotI-digested VR1012 eukaryotic expression vector (Vical Inc., San Diego, CA).

Preparation of β-galactosidase encoding FeLV pseudotype viruses. LacZ-encoding FeLV pseudotype virus used for host range analysis was generated by calcium phosphate transfection (Stratagene, La Jolla, CA) of HEK293T cells with the FeLV Env expression construct, pHit60 (MLV Gag-Pol expression construct) (41), and pMFGlacZ (14). Three days posttransfection, transfected cells were washed in phosphate-buffered saline (PBS), and fresh medium was added. The following day, culture supernatant was harvested and filtered using a 0.45-μm-pore-size filter. Filtered virus supernatant was used for infection of subsequent target cells.

LacZ-encoding FeLVs used for infection of cells expressing recombinant FeLV receptor genes was generated by transfection (PolyFect Reagent; Qiagen) of TELCeB6 cells with the pFBsalf retroviral expression vector (9) containing the FY981, FeLV-C, or FeLV-A Env gene. pFBsalf vector containing the FeLV-C or FeLV-A Env gene was generated using procedures described previously (46). pFBsalf containing the FY981 Env gene was generated by first cloning an SalI-NotI-digested FY981 Env gene (see above) into SalI-NotI-digested pBluescript vector (Stratagene) and then subsequent digest of the resultant plasmid with XbaI-ClaI and cloning of the FY981 Env gene in an XbaI-ClaI-digested pFBsalf vector. TELCeB6 cells transfected with FeLV Env expression constructs were selected with phleomycin (50 μg/ml), and resistant colonies were pooled. Virus supernatant was harvested and used for infection studies (see below).

Virus infection. Target cells were seeded in 24-well plates (1.0×10^4 cells/well) and incubated overnight at 37°C. The following day, cells were incubated with 1 ml of serially diluted LacZ-encoding FeLV-C [lacZ(FeLV-C)], lacZ(FY981), or lacZ(FeLV-A) virus supernatant for 4 h in the presence of polybrene (8 μg/ml). Virus supernatant was then replaced with fresh growth medium, and cells were allowed to incubate for a further 2 days before X-Gal (5-bromo-4chloro-3-indoyl-β-D-galactopyranoside) (Sigma-Aldrich, Canada) staining. LacZ virus infection titers were determined by counting the number of blue CFU, and titers were expressed as the number of CFU obtained per milliliter of virus supernatant.

Cloning of the porcine FeLV receptor homologues. Total RNA from porcine ST-IOWA cells was isolated using an RNAeasy kit (Qiagen), and total cDNA was generated using the Stratagene cDNA synthesis kit. Porcine FLVCR1 (poFLVCR1) cDNA was amplified by PCR using human FLVCR1 (huFLVCR1)-specific primers (upstream primer, 5′-TATCTCGAGATGGCGCGG CCAGACGATGAG-3′; downstream primer, 5′-TCAAGCGTAATCTGGTAC GTCGTATGGGTAAATTGCTGATTCTGACTGCTT-3′). The downstream primer contained additional sequences encoding the hemagglutinin epitope YP YDVPDYA (see underlined sequence) for protein detection. Porcine THTR1 (poTHTR1) cDNA was isolated using primers specific to the poTHTR1 gene identified in the NCBI database (http://www.ncbi.nlm.nih.gov/genome/guide /pig/). The primers used were the following: upstream primer, 5′-GATGTGCC CGGCCCGGTGTCTCGG-3′; downstream primer, 5′-TCAAGCGTAATCTG GTACGTCGTATGGGTAAGTGGTCACTTGAGAACGTGA-3′, containing a hemagglutinin (HA) epitope tag. poFLVCR2 cDNA was isolated by PCR using primers specific to cDNA encoding huFLVCR2 (7) transmembrane (TM) segments 1 and 12 (TM1 primer, 5′-TACTCGCTGGTCAACGCCTTTCAGTGG-3′; TM12 primer, 5′-AATGCAGTGAGGGCTGCTCCAAGAGTAAGGAAC-3′). The remaining 5′ and 3′ poFLVCR2 sequence was isolated using 5′ and 3′ rapid amplification of cDNA ends technique (Stratagene).

The porcine FeLV receptor homologues were cloned in the pFBneo retroviral vector (Stratagene). HEK293T cells were then transfected with the subsequent expression vectors, the MLV Gag-Pol expression vector, and phCMV-VSVG (expression vector containing cDNA encoding vesicular stomatitis virus G protein [VSV-G] envelope, provided by Eyal Grunebaum, Hospital for Sick Children, Toronto, Canada) to generate VSV pseudotype viruses carrying FeLV receptor sequences. VSV pseudotype virus carrying HA-tagged huFLVCR1, huFLVCR2, or feTHTR1 sequence in the pFBneo vector were also generated as described above. Virus supernatant from transfected cells was then used to infect CHO and porcine ST-IOWA cells, and transduced cells were selected using G418 (Geneticin sulfate; Invitrogen). Pooled resistant cells were then tested for susceptibility to LacZ-encoding FeLVs.

Protein analysis. Approximately 1×10^7 receptor-expressing cells grown in 75-cm^2 tissue culture flasks were lysed using 200 μl of cell lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], 5 mg/ml sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) at 4°C for 10 min. Cell genomic DNA was pelleted by centrifugation at $13,000 \times g$ for 10 min at 4°C. Cell lysate supernatant was either stored at −80°C or used for protein analysis. Approximately 100 μg of total protein was run on a 10% SDS-polyacrylamide gel, and proteins were subsequently transferred to a nitrocellulose membrane (Pall, Pensacola, FL). HA-tagged proteins were detected by incubation of nitrocellulose membranes with 1:1,000 diluted anti-HA horseradish peroxidase (HRP) monoclonal antibody (Sigma-Aldrich). Signals were detected using chemiluminescence reagent (Perkin Elmer, Boston, MA), followed by exposure to Kodak Biomax MR film. For loading control of cell lysate samples, the nitrocellulose membrane was incubated with anti-actin monoclonal antibody diluted 1 in 1,000 (Sigma-Aldrich), followed by goat anti-mouse HRP (Sigma-Aldrich) diluted 1 in 1,000. Cell membrane samples were prepared from cells grown to confluence in 150-mm diameter tissue culture plates. Cells were initially washed with PBS and then resuspended in 3 ml of cold membrane lysis buffer (20 mM Tris [pH 7.4], 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM aprotinin). The cells were scraped from the tissue culture dish using a cell scraper and then homogenized using a Dounce homogenizer. The nuclear fraction was pelleted by centrifugation at $1,000 \times g$ for 20 min at 4°C. Membrane fractions were pelleted by centrifugation of the nucleus-free supernatant at 30,000 rpm for 1 h at 4°C in a Beckman SW41 rotor. The membrane pellet was resuspended in 50 μl of PBS. A total of 25 μl of membrane sample was run on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, and HA-tagged receptor protein was detected as described above. For loading control, the remaining 25 μl of membrane sample was run on another 10% gel and transferred to a nitrocellulose membrane, which was subsequently incubated with a 1:2,000 dilution of monoclonal antibody against the α-subunit of the sodium potassium ATPase membrane protein (Sigma-Aldrich) and then with goat anti-mouse HRP antibody.

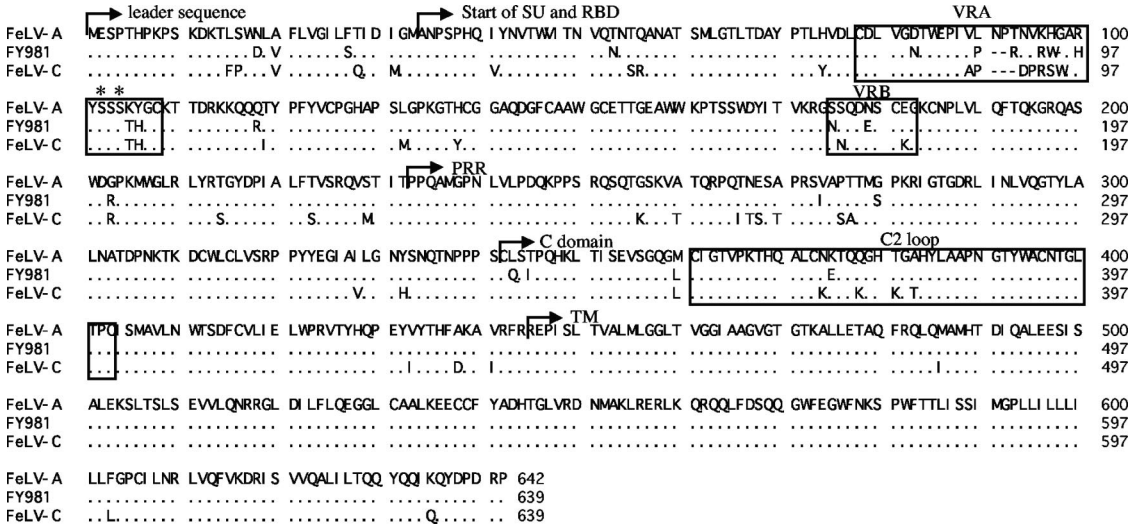

FIG. 1. The respective receptor-binding domain (RBD), PRR, C domain, and the TM region of the FeLV envelopes are shown for FeLV-A/Glasgow, FY981 variant I, and FeLV-C/Sarma Env proteins. Also shown are the variable regions VRA and VRB and the C2 loop located in the C domain. Both VRA and the C2 loop have been shown to contain residues critical for receptor binding. Asterisks above serine 99 and serine 101 indicate the residues that were mutated to prolines in variant II clones of FY981 Envs. Dots indicate identical residues, and dashes indicate spaces that were introduced for alignment. The Env sequences were aligned using the BioEdit sequence alignment program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

Generation of soluble FeLV-A, FeLV-C, and FY981 SU proteins and SU binding assay. A pCS-FSCHA expression construct containing FeLV-C/Sarma SU cDNA fused in frame with a double HA epitope was kindly provided by Julie Overbaugh. This construct was generated as described previously by Sugai and colleagues (43). FY981 SU and FeLV-A SU expression constructs were generated by first amplifying FY981 and FeLV-A Env cDNA using the upstream primer 5′-GGACGTCGGAGGAAGCTTGAT-3′ and downstream primer 5′-GGGGGAGCTCGTAAATATATTCGGGTTGATG-3′ and subsequently cloning the amplified SU cDNAs in a SacI-digested pCS-HA vector in frame with the double HA epitope. HEK293 cells, seeded at 1×10^6 cells in a 100-mm culture dish 1 day prior to transfection, were subsequently transfected with 10 μg of FeLV SU expression construct using PolyFect transfection reagent. Two days posttransfection, supernatant was harvested and filtered using a 0.45-μm-pore-size filter. The HA-tagged FeLV SU proteins were subsequently stored at −80°C and used for envelope binding studies.

CHO and ST-IOWA cells stably expressing FLVCR1, FLVCR2, or THTR1 were treated with a cell dissociation buffer (Invitrogen) to dislodge cells. Approximately 1×10^6 cells were used for each binding assay. The cells were first incubated with 1 ml of the respective HA-tagged FeLV SU in the presence of polybrene (8 μg/ml) for 30 min at 37°C. Cells were then centrifuged at 4,000 rpm for 3 min. All subsequent spins were carried out at 4,000 rpm for 3 min. Cells were then washed two times with cold PBS containing 2% FBS (2% PFBS). Target cells were then incubated with 100 μl of 2% PFBS containing a 1:200 dilution of monoclonal HA.11 antibody (Covance, Berkley, CA) on ice for 30 min. Cells were washed again two times with 2% PFBS before incubation with 100 μl of PFBS containing a 1:25 dilution of donkey anti-mouse antibody conjugated to fluorescein isothiocyanate (1 mg/ml) (Sigma) for 30 min on ice. Cells were then analyzed for envelope binding by flow cytometry (Beckman Coulter, Mississauga, Ontario, Canada).

Immunoprecipitation of soluble SU protein. Approximately 1 ml of supernatant containing HA-tagged FY981 SU, FeLV-C SU, or FeLV-A SU protein was precleared with 100 μg/ml of a 50% protein G-Sepharose (GE Healthcare Biosciences AB) suspension for 3 h at 4°C. The supernatants were then immunoprecipitated for 2 h at 4°C with bound C11D8 anti-gp70 antibody-bead complex for isolation of full-length soluble SU proteins. Immunoprecipitates were washed three times in PBS containing 0.1% Triton X-100 and 0.1% NP-40. The bound SU proteins were eluted in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer and heated to 100°C. Approximately 25 μl of sample was analyzed by Western blot analysis using a 1:100 dilution of anti-HA antibody conjugated to HRP (Sigma).

Expression of FLVCR2 siRNA in porcine ST-IOWA cells. Two 25-base length small interfering RNA (siRNA) oligonucleotides, S1 (FLVCR2HSS124723) and S2 (FLVCR2HSS124724), specific to human FLVCR2 (siRNAs are available from Invitrogen) were used to knock down FLVCR2 expression. To validate the specificity of the FLVCR2 siRNAs, CHO cells expressing HA-tagged huFLVCR1 or huFLVCR2 were seeded (1×10^5) in a 12-well plate. The next day, cells were transfected with 100 pmol of S1 or S2 or with control scrambled siRNA (Invitrogen) using a Lipofectamine RNAi Max kit (Invitrogen). Transfected cells were incubated for a further 2 days before preparation of cell lysates and analysis of HA-tagged FLVCR1 and FLVCR2 protein expression by Western blotting (see above). Porcine ST-IOWA cells expressing scrambled, S1, or S2 siRNA were generated by transfection of ST-IOWA cells with 100 pmol of siRNA oligonucleotides. At 2 days posttransfection, cells were infected with serial dilutions of β-galactosidase encoding FY981 pseudotype virus, and infected cells were stained 2 days later as described above.

Nucleotide sequence accession numbers. The FY981 FeLV Env and poFLVCR1, poFLVCR2, and poTHTR1 cDNA and protein sequences have been deposited in the GenBank database under accession numbers FJ436991, FJ436992, FJ436993, and FJ436994, respectively.

RESULTS

Isolation and characterization of the novel FY981 FeLV envelope sequence. To investigate potential FeLV variants that arise during the emergence of pathogenic FeLV-C in infected cats, we amplified FeLV envelope (Env) sequences from the primary FY981 FeLV isolate that was previously derived from a domestic cat diagnosed with pure red cell aplasia (Jarrett, unpublished). The FY981 primary isolate had previously been characterized by interference studies as containing a mixture of FeLV subgroups A, B, and C (Jarrett, unpublished). We first propagated the FY981 primary isolate on the nonrestrictive feline FEA embryonic fibroblast cell line to allow growth of all FeLVs. Full-length FeLV Env sequences were then amplified by PCR from the FY981-infected FEA cells (see Materials and Methods). Seven FY981 FeLV Env clones were subsequently isolated and sequenced. Five of the seven FeLV Envs were identical to each other and were termed variant I, whereas the remaining two FeLV Envs contained an additional serine-to-

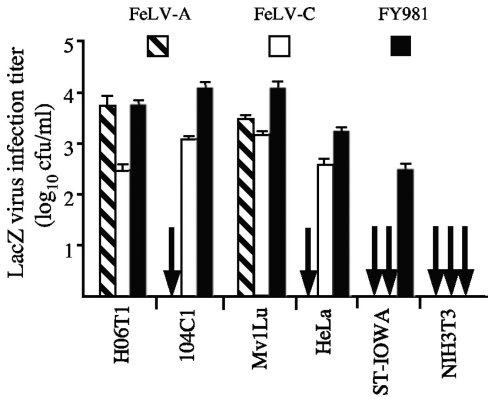

FIG. 2. LacZ-encoding virus bearing either FY981, FeLV-C, or FeLV-A Env was generated by cotransfection of HEK293 cells with MLV Gag-Pol, LacZ, and Env expression constructs. Supernatants were harvested, and infection titers were tested on a panel of cell lines. Titers are represented as the number of CFU per milliliter of virus supernatant, and values are averages of three independent infection studies. The cell lines tested are feline kidney H06T1, guinea pig 104C1, mink lung Mv1Lu, human cervical carcinoma HeLa, porcine testes ST-IOWA, and murine NIH 3T3. Arrows indicate zero infection titers. Standard deviation is shown for each infection.

proline change at positions 99 and 101 (Fig. 1) and were termed variant II. The amplified FY981 FeLV Envs were more closely related to the prototypical FeLV-A/Glasgow Env sequence (42) (96% amino acid identity) than to the prototypical FeLV-C/Sarma Env sequence (37) (91% amino acid identity), particularly in the proline-rich region (PRR), C domain, and TM region (Fig. 1). However, the variable region A (VRA) sequence of the FY981 Env clones contained a 3-amino-acid deletion and a TH sequence that was characteristic of a FeLV-C Env (Fig. 1). Previous studies have demonstrated that amino acid alterations in VRA alone are sufficient to confer the subgroup C phenotype upon FeLV-A (6, 38). Based on the sequence similarity of FY981 VRA to FeLV-C VRA, we predicted that the FY981 Env clones would be subgroup C.

FY981 FeLV uses the FeLV-A and FeLV-C receptors for infection. To ascertain the host range of the FY981 Env, we generated β-galactosidase (LacZ)-encoding pseudotype viruses bearing a representative Env clone from variant I or variant II and tested the ability of the pseudotype viruses to infect feline H06T1, guinea pig 104C1, mink lung Mv1Lu, human HeLa, porcine ST-IOWA, and murine NIH 3T3 cells. However, we were unable to generate an infectious pseudotype virus using the variant II FY981 Env, and hence a representative clone from variant I FY981 Env was characterized further (Fig. 1). As shown in Fig. 2, the host range of FY981 FeLV was similar to that of FeLV-C, capable of infecting feline, mink, human, and guinea pig cells, whereas FY981 and FeLV-C did not infect murine NIH 3T3 cells. Infection of guinea pig cells is a characteristic of a subgroup C virus. Interestingly, FY981 FeLV also infected porcine ST-IOWA cells, which were resistant to FeLV-A and FeLV-C (Fig. 2).

To ascertain the host receptor used by FY981 FeLV, we tested the ability of the lacZ(FY981) virus to use huFLVCR1 for infection. lacZ(FeLV-C) and lacZ(FeLV-A) pseudotype viruses were used as controls. lacZ(FY981) and lacZ(FeLV-C) infection titers on human TE671 cells were on average 2.3×10^5 CFU/ml and 3.3×10^6 CFU/ml, respectively, whereas the lacZ(FeLV-A) infection titer on murine MDTF cells overexpressing the feline FeLV-A receptor THTR1 (feTHTR1) (23) was 1.8×10^5 CFU/ml. We initially tested FY981 pseudotype virus infection on NIH 3T3 cells expressing huFLVCR1. However, we have recently found that infection by all FeLV pseudotype viruses is weak on NIH 3T3 cells expressing the respective FeLV receptors, despite efficient expression of FeLV receptors (unpublished data). We also tested FY981 infection on MDTF cells, which are resistant to FeLV-A and FeLV-C (7, 23), but found that parental MDTF cells had a relatively high background FY981 infection titer of FY981

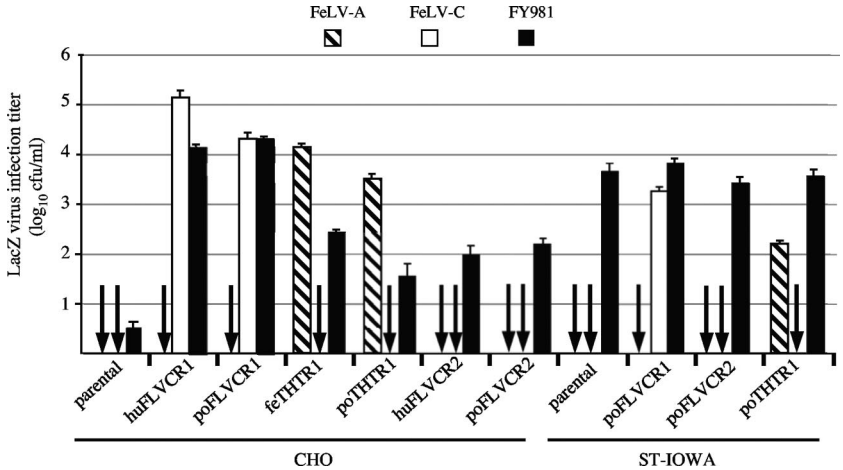

FIG. 3. CHO cells were stably transduced with human (hu), feline (fe), or porcine (po) FeLV receptor expression constructs using VSV pseudotype virus. ST-IOWA cells were also transduced with VSV pseudotype virus with porcine FeLV receptor expression constructs. Transduced cells were selected using G418, and resistant cells were pooled and tested for susceptibility to lacZ(FY981), lacZ(FeLV-C), or lacZ(FeLV-A). Parental CHO and ST-IOWA cells were included. Titers are averages of three independent infections and are represented as the number of CFU per milliliter of virus supernatant. Standard deviation is shown for each infection. Arrows indicate zero infection titers.

(approximately 10^2 CFU/ml). Therefore, we tested FY981 host receptor use in CHO cells, which had a low background FY981 infection titer (5 CFU/ml) (Fig. 3). CHO cells were also resistant to FeLV-A and FeLV-C. We first tested the ability of FY981 to infect CHO cells expressing huFLVCR1 (CHO/huFLVCR1). The huFLVCR1 expression construct and all subsequent FeLV receptor constructs were introduced in CHO cells by infection using VSV pseudotype virus (see Materials and Methods). As expected, CHO/huFLVCR1 cells were highly susceptible to FeLV-C but resistant to FeLV-A (Fig. 3). CHO/huFLVCR1 cells were also highly susceptible to FY981 (Fig. 3), suggesting that FY981 is an FeLV-C variant. Because of the close sequence identity of FY981 Env to FeLV-A Env (Fig. 1), we also tested the ability of FY981 pseudotype virus to infect CHO cells expressing feTHTR1 (CHO/feTHTR1). As expected CHO/feTHTR1 cells were highly susceptible to FeLV-A but resistant to FeLV-C (Fig. 3). Interestingly, we found that CHO/feTHTR1 cells were susceptible to FY981 infection, with an average titer of 3.5×10^2 CFU/ml. This represented a 70-fold increase in FY981 infection compared to FY981 infection on parental CHO cells, thus suggesting that FY981 can also use the FeLV-A receptor for infection. Together, our results suggest that FY981 can use both the FeLV-A and FeLV-C receptors for infection.

Using flow cytometry, we next tested the ability of the soluble SU protein of FY981 tagged with a double HA epitope to bind to CHO/huFLVCR1 and CHO/feTHTR1 cells. HA-tagged FeLV-C SU and FeLV-A SU were also used in binding assays as controls. We first quantitated the amount of FY981 SU, FeLV-C SU, and FeLV-A SU protein present in 1 ml of SU supernatant that we used for each binding assay. Relative to FY981 SU, there was approximately 40% less FeLV-C SU and 40% more FeLV-A SU in 1 ml of supernatant (Fig. 4B). As depicted in Fig. 4A, we observed significant binding of FeLV-C SU and FeLV-A SU to their respective receptor-expressing CHO cells as determined by an increase in fluorescence intensity (Fig. 4A, histogram of binding on CHO/huFLVCR1 and CHO/feTHTR1). We also observed significant binding of FY981 SU to CHO/huFLVCR1 cells (Fig. 4), consistent with the idea that huFLVCR1 is an efficient receptor for FY981 (Fig. 3). However, we did not detect binding of FY981 SU to CHO/feTHTR1 cells even though these cells are sensitive to FY981 infection. Interestingly, we observed binding of FeLV-A SU to parental CHO cells (Fig. 4A), which are resistant to FeLV-A infection (Fig. 3). Binding of FeLV-A SU was observed on all FeLV receptor-expressing CHO cells (Fig. 4A).

The porcine homologues of FLVCR1 and THTR1 are not responsible for the susceptibility of ST-IOWA cells to FY981. To investigate the differential susceptibility of porcine ST-IOWA cells to FY981 and FeLV-C (Fig. 2), we first isolated poFLVCR1 and tested the ability of this protein to mediate FeLV infection. The poFLVCR1 protein consists of 555 amino acids and shares 84% amino acid identity to huFLVCR1 (Fig. 5). Interestingly, extracellular loop 1 (ECL1) sequence and ECL6 residue 487 in poFLVCR1 differ from huFLVCR1 sequence (Fig. 5, ECL6). These two regions in huFLVCR1 have previously been shown by our group to be important for FeLV-C receptor function (7). To ascertain whether sequence variations in poFLVCR1 ECL1 and residue 487 were responsible for FY981 susceptibility and FeLV-C resistance of porcine ST-IOWA cells, we tested the FeLV susceptibility of CHO cells expressing poFLVCR1 (CHO/poFLVCR1). As shown in Fig. 3, we found that CHO/poFLVCR1 cells were highly susceptible to both FY981 and FeLV-C, suggesting that poFLVCR1 is a functional receptor for both FeLVs. Consistent with the infection data, we observed binding of FeLV-C SU and FY981 SU to poFLVCR1 (Fig. 4A). However, binding of FeLV-C SU and FY981 SU to CHO/poFLVCR1 cells was weaker than binding to CHO/huFLVCR1 cells. To ascertain whether the receptor expression level was responsible for the reduced SU binding, we determined FLVCR1 protein expression in crude membrane preparations from CHO/huFLVCR1 and CHO/poFLVCR1 cells. The huFLVCR1 and poFLVCR1 proteins were approximately 65 to 70 kDa in size (Fig. 6A), which was consistent with our previously reported size of a glycosylated form of FLVCR1 (7). We found that membrane expression of poFLVCR1 was significantly lower than membrane expression of huFLVCR1 (Fig. 6A, crude membrane blot). The reduced membrane expression was not attributed to reduced cellular expression of poFLVCR1 (Fig. 6A, cell lysate blot). Thus, the reduced FeLV-C SU and FY981 SU binding to CHO/poFLVCR1 was likely attributed to low poFLVCR1 membrane expression. We also isolated and tested the receptor function of poTHTR1, which shares 90% amino acid identity with feTHTR1 (data not shown). We found that CHO/poTHTR1 cells were susceptible to FeLV-A but resistant to FeLV-C (Fig. 3), suggesting that poTHTR1 is a functional FeLV-A receptor. We also found that CHO/poTHTR1 cells were weakly susceptible to FY981, with infection titers being consistently 10-fold greater than titers observed on parental CHO cells (Fig. 3). Membrane expression of poTHTR1 was relatively equivalent to that of feTHTR1 (Fig. 6A). This suggests that poTHTR1 is a weak receptor for FY981. Subsequent SU binding analysis revealed strong binding of FeLV-A SU and weak binding of FY981 SU to CHO/poTHTR1 cells (Fig. 4), which correlated with the strong and weak susceptibility of CHO/poTHTR1 cells to FeLV-A and FY981, respectively (Fig. 3). Surprisingly, we observed binding of FeLV-C SU to CHO/poTHTR1 cells even though these cells were resistant to FeLV-C infection.

To further test the receptor function of poFLVCR1 and poTHTR1, we overexpressed the respective FeLV receptors in porcine ST-IOWA cells and tested the susceptibility of the cells to FY981, FeLV-C, and FeLV-A. Overexpression of poFLVCR1 in ST-IOWA cells (ST/poFLVCR1) rendered the cells susceptible to FeLV-C (Fig. 3), whereas no change was observed in FY981 and FeLV-A infections compared to infection on parental ST-IOWA cells. Similarly, overexpression of poTHTR1 in ST-IOWA cells (ST/poTHTR1) cells rendered the cells susceptible to FeLV-A (Fig. 3). Our findings suggest that the endogenous poFLVCR1 and poTHTR1 are functional FeLV-C and FeLV-A receptors, respectively, when overexpressed in porcine cells. We also tested binding of FeLV SUs to ST-IOWA, ST/poFLVCR1, and ST/poTHTR1 cells (Fig. 4A). Despite increased susceptibility of ST/poFLVCR1 to FeLV-C, we did not detect binding of FeLV-C SU and observed only weak binding of FY981 SU to these cells. Similarly, although we observed background FeLV-A SU binding to parental ST-IOWA cells (Fig. 4A), we did not observe an increase in FeLV-A SU binding to ST/poTHTR1 cells even

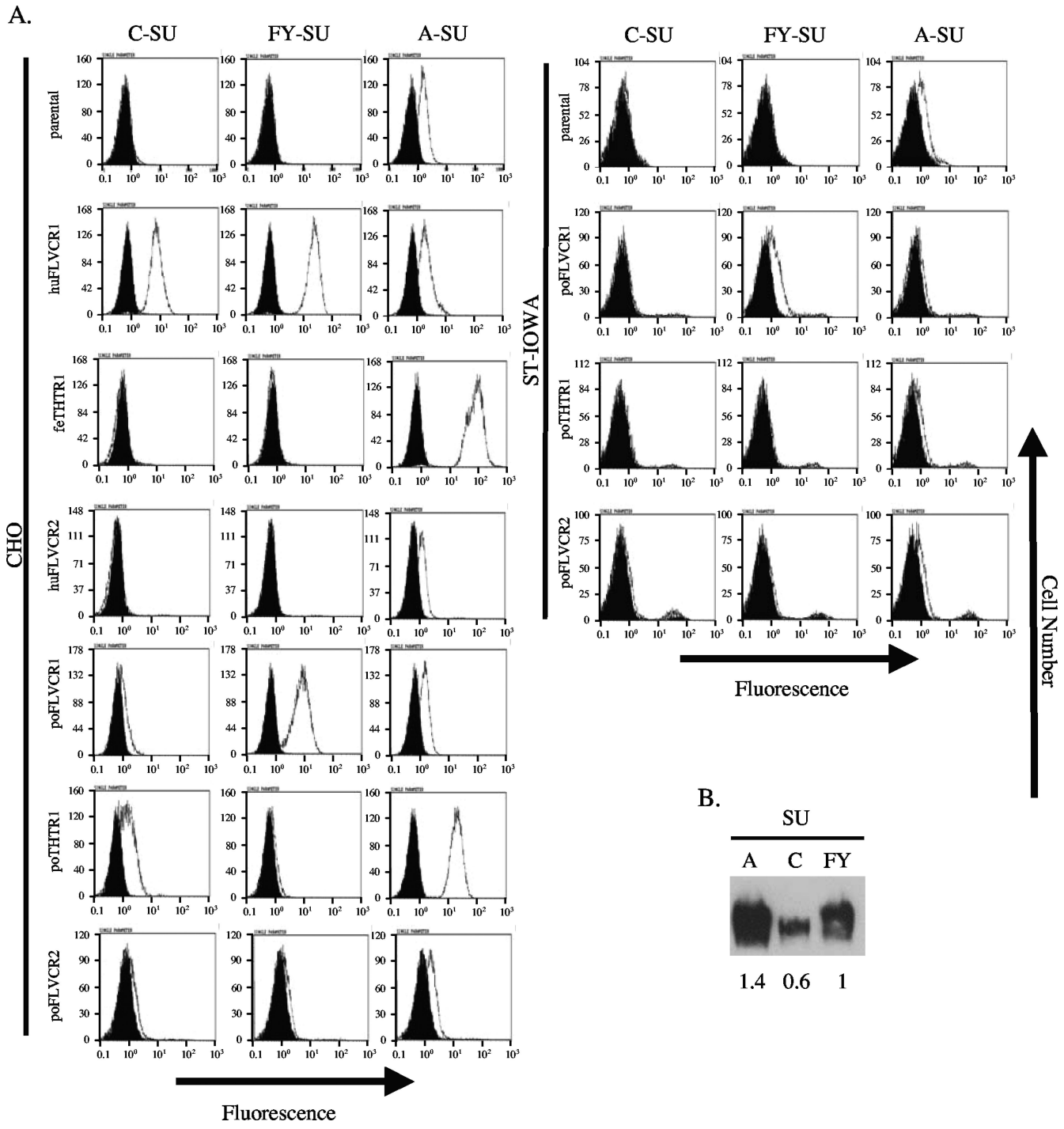

FIG. 4. (A) Receptor-expressing cells were incubated with (white histogram) or without (black histogram) FeLV-C SU (C-SU), FY981 SU (FY-SU), or FeLV-A SU (A-SU) proteins tagged with a double HA epitope. Bound SU protein was detected using mouse anti-HA antibody (HA.11) and fluorescein-conjugated donkey anti-mouse and analyzed by flow cytometry. An increase in fluorescence (white histogram) represents SU binding. (B) Soluble FeLV-A (A), FeLV-C (C), or FY981 (FY) SU proteins in 1 ml of SU supernatant. Relative amount of protein (determined using ImageJ software) is shown below the blot.

though these cells are sensitive to FeLV-A infection. Subsequent analysis of membrane expression of the porcine FeLV receptors showed that poFLVCR1 was efficiently expressed (Fig. 6B), whereas poTHTR1 was weakly expressed. Thus, the nondetectable binding of FeLV-C SU to ST/poFLVCR1 cells is likely attributed to lower levels of soluble FeLV-C SU protein used in the binding assay (Fig. 4B), whereas the nondetectable binding of FeLV-A SU to ST/poTHTR1 cells could be attributed to low membrane expression of poTHTR1. Taken together, our observation that ST-IOWA cells become suscep-

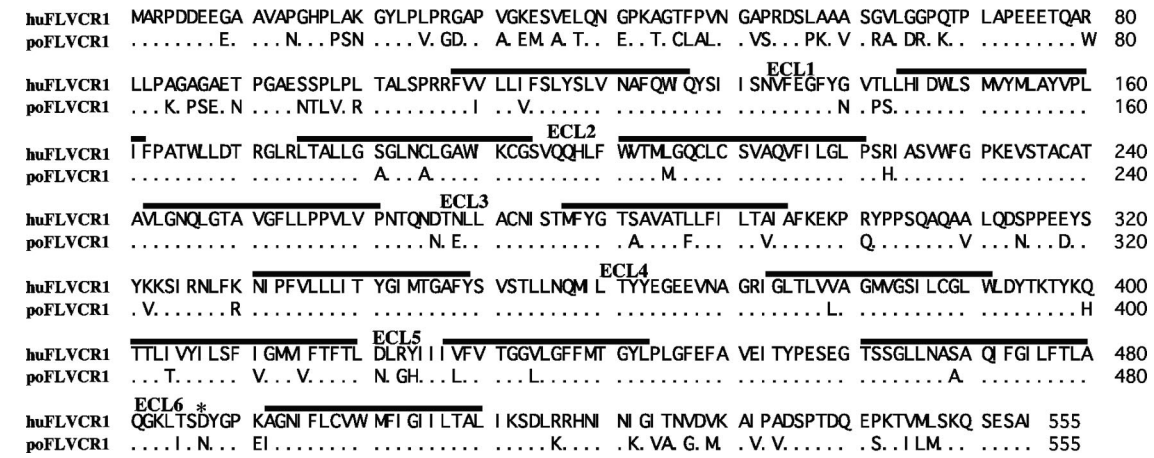

FIG. 5. The predicted TM-spanning segments of huFLVCR1 and poFLVCR1, determined using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) are indicated by a line above the amino acid sequences. Presumptive ECLs are indicated above the sequences. The asterisk indicates residue 487 in ECL6 that had previously been shown to be critical for receptor function (7). Dots represent identical residues. The FLVCR1 sequences were aligned using the BioEdit sequence alignment program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

tible to FeLV-A and FeLV-C when the endogenous poFLVCR1 and poTHTR1 are overexpressed suggests that the endogenous FeLV receptors in parental ST-IOWA cells are expressed at a subthreshold level that prevents FeLV infection. This raised the possibility that FY981 infection of ST-IOWA cells was being mediated by an alternative porcine receptor.

The FLVCR1-related protein FLVCR2 is a functional receptor for FY981. Previous studies have shown that gammaretroviruses often use alternative receptors that are highly related in sequence and in cellular function to their cognate receptors (reviewed in references 31 and 44). We had previously isolated the FLVCR1-related sequence FLVCR2 from human cells. We had further shown that huFLVCR2 is nonfunctional as an FeLV-C receptor but becomes functional by introduction of a single residue mutation in ECL6 of huFLVCR2 (7). This raised the possibility that FLVCR2 could be the alternative FY981 receptor. To ascertain whether FLVCR2 could mediate FY981 infection, we first tested FY981 susceptibility of CHO cells expressing huFLVCR2 (CHO/huFLVCR2). As shown in Fig. 6B, membrane expression of huFLVCR2 was significantly greater than FLVCR1 and THTR1 membrane expression. The 55-kDa and 40-kDa sizes of the huFLVCR2 protein were consistent with the previously reported size of this protein (7). Interestingly, we found that CHO/huFLVCR2 cells were susceptible to FY981 but resistant to FeLV-A and FeLV-C (Fig. 3), which correlated with the infectivity of porcine cells to FY981, FeLV-A, and FeLV-C (Fig. 3). Our finding suggested that FY981 infection of ST-IOWA cells was mediated by porcine poFLVCR2. To test this, we isolated and then assessed the receptor function of poFLVCR2. The poFLVCR2 protein (549 amino acids) shares 83% amino acid identity to huFLVCR2 (526 amino acids) with strong sequence conservation in the presumptive ECLs (data not shown). In agreement with FeLV infections of CHO/huFLVCR2 cells, we found that CHO/poFLVCR2 cells were susceptible to FY981 but resistant to FeLV-A and FeLV-C (Fig. 3). We also tested binding of FeLV SUs on FLVCR2-expressing cells. We detected binding of FY981 SU and FeLV-C SU, albeit weak, to CHO/poFLVCR2 cells but did not detect binding of these SUs on CHO/huFLVCR2 cells (Fig. 6A). We also overexpressed poFLVCR2 in ST-IOWA cells (Fig. 6B), but we did not observe an enhancement in FY981 infection titer (Fig. 3), nor did we detect FY981 SU binding on these cells (Fig. 4A). Subsequent analysis of receptor expression in ST/poFLVCR2 cells showed significant membrane expression of poFLVCR2 (Fig. 6B). Interestingly, we observed a 110-kDa protein in addition to the expected 55-kDa and 40-kDa proteins in the ST/poFLVCR2 sample. This higher-molecular-mass protein could represent a glycosylated form or a dimer of poFLVCR2.

Expression of FLVCR2-specific siRNA in ST-IOWA cells disrupts FY981 infection. To provide further evidence that FY981 infection of ST-IOWA cells is mediated by poFLVCR2, we tested FY981 susceptibility of ST-IOWA cells transiently transfected with FLVCR2-specific siRNA. We tested the ability of two siRNA oligonucleotide sequences (S1 and S2) specific to huFLVCR2 (siRNAs specific to poFLVCR2 were not available for this study) to disrupt FY981 infection of porcine ST-IOWA cells. To validate the specificity of FLVCR2 siRNAs, we transiently expressed the S1 and S2 siRNAs or a control scrambled (Scr) siRNA in CHO/huFLVCR2 and CHO/huFLVCR1 cells and subsequently analyzed expression of HA-tagged huFLVCR2 and huFLVCR1 proteins. As shown in Fig. 7A, we found that expression of S1 and S2 reduced expression of HA-tagged huFLVCR2 by 25% and 35%, respectively, relative to huFLVCR2 expression in cells transfected with Scr siRNA, whereas expression of huFLVCR1 was not disrupted. This finding shows that S1 and S2 siRNAs specifically disrupt FLVCR2 expression. We then transiently expressed an S1, S2, or Scr siRNA in ST-IOWA cells and tested the susceptibility of these cells to FY981. The S1 and S2 siRNAs showed a respective 93% match (23/25 nucleotide matches) and 64% match (19/25 nucleotide matches) with the corresponding sequences in poFLVCR2. As shown in Fig. 7B, relative to the FY981 infection titer on ST-IOWA cells with Scr siRNA, we observed a 76% and 65% reduction in FY981 infection titer on ST-IOWA cells with S1 and S2 siRNAs,

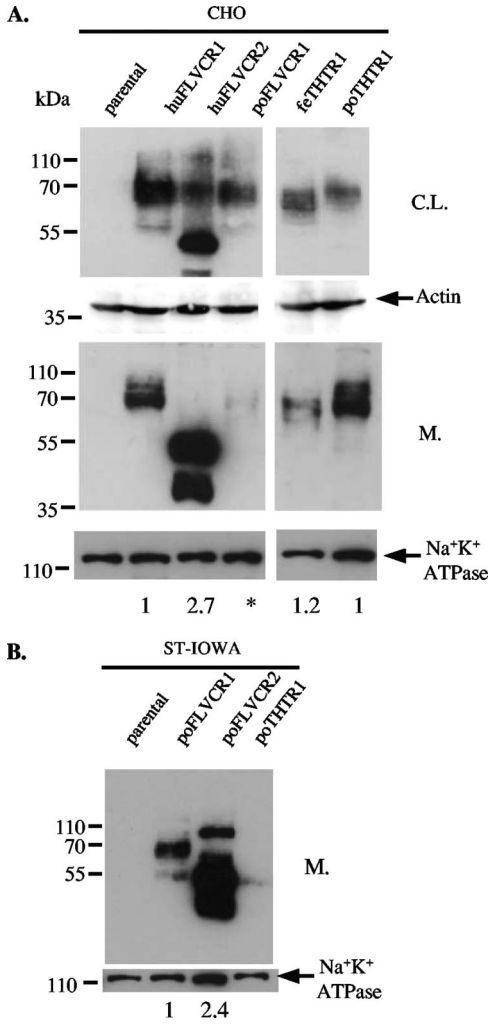

FIG. 6. (A) FeLV receptor protein expression in cell lysate (CL) and crude membrane (M) prepared from CHO cells transduced with VSV pseudotype virus carrying feline (fe), human (hu), or porcine (po) FeLV receptor (FLVCR1, FLVCR2, or THTR1) constructs. Receptor proteins were tagged with an HA epitope, and proteins were detected using an anti-HA HRP-conjugated monoclonal antibody. The loading control for actin and Na^+K^+ ATPase are shown. The relative membrane receptor expression is shown below the ATPase blot and was determined using ImageJ Software (see Materials and Methods). Membrane expression is relative to huFLVCR1. The asterisk denotes low membrane expression. (B) FeLV receptor protein expression in crude membrane (M) prepared from porcine ST-IOWA cells expressing HA-tagged poFLVCR1, poFLVCR2, and poTHTR1. The Na^+K^+ ATPase loading control is shown with relative membrane expression.

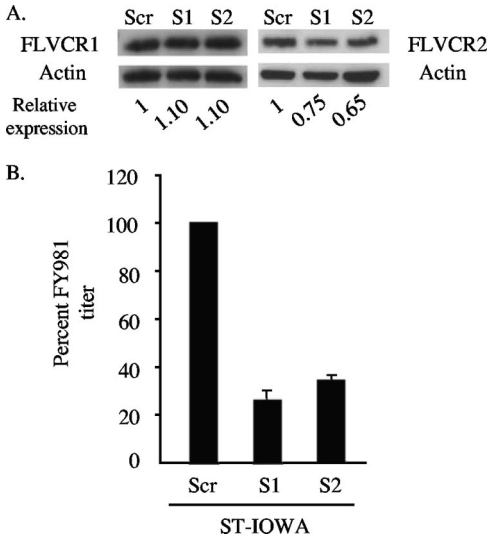

FIG. 7. (A) Validation of FLVCR2 knockdown by FLVCR2 siRNA. CHO/huFLVCR2 or CHO/huFLVCR1 cells were transiently transfected with huFLVCR2-specific siRNAs S1 or S2 or with Scr siRNA, and expression of the HA-tagged huFLVCR1 or huFLVCR2 proteins was analyzed by Western blotting. (B) FY981 infection titer in porcine ST-IOWA cells transiently expressing Scr, S1, or S2 siRNAs. The percent FY981 infection titer is shown relative to infection observed on ST-IOWA cells expressing Scr siRNA. Infection titers are averages of three infection experiments. Standard deviation bars are shown.

respectively (Fig. 7B). This finding provides further evidence that FY981 infection of ST-IOWA cells is mediated by poFLVCR2.

DISCUSSION

In this study, we report the characterization of a novel FeLV Env (FY981 Env) that can individually use multiple receptors for infection. We show that pseudotype virus bearing the FY981 Env uses the FeLV-C receptor FLVCR1 and the FeLV-A receptor THTR1 for infection. We further show that FY981 virus has an expanded host range to infect porcine ST-IOWA cells, and we provide evidence that the expanded host range is attributed to the use of the FLVCR1-related protein FLVCR2. Our findings provide the first report of an FeLV and of a gammaretrovirus that uses three distinct receptors for infection.

The FY981 primary isolate was previously derived from an FeLV-infected anemic cat and was characterized by interference studies as containing a mixture of subgroups A, B, and C (Jarrett, unpublished). However, we were unable to amplify FeLV-A or FeLV-B Env sequences from the primary isolate but amplified two FY981 FeLV Env variants (variants I and II) that differed by two residues (Fig. 1). Interestingly, the amplified FY981 Envs show strong sequence identity to FeLV-A Env in the PRR, C domain, and TM but contain a VRA sequence that partially resembles the VRA sequence from the prototypical FeLV-C/Sarma Env (Fig. 1). Because the VRA sequence predominantly determines receptor specificity, the resemblance of FY981 VRA to FeLV-C VRA suggests that FY981 is a subgroup C FeLV. Consistent with this, we found that FY981 pseudotype virus infected guinea pig cells (Fig. 2) and CHO cells expressing huFLVCR1 (Fig. 3). Interestingly, we found that FY981 also infected CHO cells expressing feTHTR1 (Fig. 3), suggesting that FY981 can also use the FeLV-A receptor for infection. The use of both huFLVCR1 and feTHTR1 as receptors by FY981 is consistent with the idea that FY981 Env is a hybrid FeLV-A/FeLV-C Env.

In this study, we found that FY981 had an expanded host range to infect porcine ST-IOWA cells, which are naturally resistant to FeLV-A and FeLV-C. Our results suggest that the endogenous poFLVCR1 and poTHTR1 are not responsible

for mediating FY981 infection of ST-IOWA. We found that CHO/poFLVCR1 cells were highly susceptible to both FY981 and FeLV-C, and overexpression of ST/poFLVCR1 cells rendered the cells susceptible to FeLV-C infection (Fig. 3). This finding suggests that the poFLVCR1 is a functional receptor for both FY981 and FeLV-C when overexpressed. Similar receptor function analysis of CHO/poTHTR1 and ST/poTHTR1 cells suggests that poTHTR1 is a functional receptor for FeLV-A and for FY981, albeit weakly. Thus, the resistance of porcine cells to FeLV-C and FeLV-A is not caused by an inherent defect in the endogenous receptors. Our findings suggest that the endogenous poFLVCR1 and poTHTR1 are expressed at a subthreshold level in ST-IOWA cells that prevents FeLV-C and FeLV-A infection, respectively. Our conclusions are consistent with our previously published report (45) investigating the receptor function of the endogenous MDTF FLVCR1 and CHO Pit1 receptor homologues, which render their respective parental cells sensitive to FeLV infection when they are overexpressed. Thus, because of the subthreshold expression of poFLVCR1 and poTHTR1, we propose that the infection of ST-IOWA cells by FY981 is not mediated by FLVCR1 and THTR1. We reason that if the endogenous poFLVCR1 or poTHTR1 proteins are responsible for mediating FY981 infection of ST-IOWA cells, then we would expect ST-IOWA cells to be susceptible to FeLV-C and FeLV-A, respectively. We cannot completely exclude the possibility that FY981 infection of ST-IOWA cells is mediated by poFLVCR1 or poTHTR1. It is conceivable that FY981 binds more efficiently to the FeLV receptors than FeLV-C and FeLV-A and that this binding is sufficient to allow FY981 infection even at subthreshold receptor expression levels. However, our infection data (Fig. 3) and FLVCR2 siRNA data (Fig. 7A and B) strongly suggest that FY981 infection of ST-IOWA cells is mediated by an alternative porcine receptor, which we have identified as FLVCR2, a protein that shares sequence and topological identity to FLVCR1 (7). We previously reported that a single amino acid mutation in ECL 6 of huFLVCR2 was sufficient to render huFLVCR2 functional as an FeLV-C receptor (7). We proposed in this previous study the possibility of the existence of FeLV-C variants that could adapt to use FLVCR2 as a receptor. We now show in this study that FY981, a FeLV-C variant, uses FLVCR2 as a receptor. CHO/poFLVCR2 or CHO/huFLVCR2 cells were susceptible to FY981 but resistant to FeLV-A and FeLV-C infections, which correlated with the pattern of FeLV infectivity observed on parental ST-IOWA cells (Fig. 2 and 3). Furthermore, transient expression of FLVCR2-specific siRNAs (Fig. 7A) in ST-IOWA cells disrupted FY981 infection (Fig. 7B). The use of FLVCR2 as a receptor is consistent with previous reports that retroviruses often use structurally and functionally related proteins as receptors (reviewed in references 31 and 44). Based on the sequence and topological similarity of FLVCR2 to heme exporter FLVCR1 (35), it is tempting to speculate that the cellular function of FLVCR2 could also be to transport heme.

Our SU binding data were generally in agreement with our infection data. We observed binding of FeLV-C SU and FY981 SU to FLVCR1-expressing CHO cells. Binding of FeLV-C SU was generally weaker than FY981 SU binding to FLVCR1-expressing cells (Fig. 4A). This is likely attributed to the reduced level of FeLV-C SU protein compared to FY981 SU protein in the 1 ml of supernatant used for the binding assay (Fig. 4B). We also observed strong binding of FeLV-A SU to CHO/feTHTR1 and CHO/poTHTR1 cells, which was consistent with the susceptibility of these cells to FeLV-A (Fig. 3). However, our SU binding did provide interesting observations. We observed binding of FeLV-A SU on parental CHO cells even though these cells are resistant to FeLV-A. This could be attributed to mutations in the endogenous THTR1 in CHO cells or to posttranslational modifications in CHO cells (24) that prevent FeLV-A infection but not binding. Interestingly, we consistently observed significant binding of FeLV-C SU to CHO/poTHTR1 cells (Fig. 4) even though poTHTR1 is characterized as an FeLV-A receptor (Fig. 3). It is unclear from this study why FeLV-C SU binds to poTHTR1. Our findings raise the possibility that poTHTR1 and FLVCR1, despite belonging to different transporter families (16, 33), may share some similar extracellular motifs that allows FeLV-C SU binding to poTHTR1. Additional studies will be required to address this phenomenon. In this study, we also observed weak binding of FeLV-C SU and FY981 SU to CHO/poFLVCR2 cells (Fig. 4), yet CHO/poFLVCR2 cells were susceptible to FY981 but not to FeLV-C (Fig. 3). Together, our findings support previous observations and conclusions (13, 22) that retroviral receptor regions involved in SU binding are distinct from regions involved in virus entry. In addition to the phenomenon of binding without infection, we observed FeLV infection but no detectable SU binding on some receptor-expressing cells. Despite observing FY981 infection, we did not detect binding of FY981 SU to CHO/huFLVCR2, CHO/feTHTR1, or parental ST-IOWA cells, and we observed at best only weak binding of FY981 SU to ST/poFLVCR2 overexpressing cells (Fig. 4A). The nondetectable or weak binding of FY981 SU could be attributed to the insensitivity of our binding assay. Alternatively, it is conceivable that the use of monomeric SU protein in our binding assay may not fully represent binding of oligomeric Envs expressed on virions. Indeed, previous studies have shown that neutralizing antibodies raised to monomeric HIV-1 SU do not neutralize infection of primary HIV-1 isolates (25) and that SU antibodies that neutralize infection of primary HIV-1 isolates correlate more with binding to oligomeric HIV SU than to monomeric HIV SU (15). Inconsistency in the binding correlation between monomeric and oligomeric SUs has also been reported for feline immunodeficiency virus (48).

In conclusion, our data suggest that the FY981 Env, a novel hybrid FeLV-A/FeLV-C Env, recognizes THTR1, FLVCR1, and FLVCR2 as receptors. Our findings raise the possibility that FY981 could represent a variant that arose during the emergence of FeLV-C. The absence of prototypical FeLV-A or FeLV-C Envs from the primary FeLV isolate would suggest that the FY981 Env is the dominant Env sequence that may have out-competed FeLV-A and potentially FeLV-C. Alternatively, the FY981 Env may represent an intermediate that has yet to transition completely to an FeLV-C Env. Characterization of FeLV Envs from additional FeLV primary isolates may provide further novel insights into intermediates that arise during emergence of pathogenic FeLV-C and into the host receptors used for infection. Our findings show striking analogy to the receptor adaptation mechanism by HIV-1, in which the emergence of the highly pathogenic X4 HIV-1 involves

variants/intermediates that are dual tropic or multitropic in their coreceptor use (4, 5, 11). Taking these results together, we propose that pathogenic FeLV-C emerges in infected cats through FeLV intermediates that are multitropic in their receptor use and that this mechanism is common for other gammaretroviruses.

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